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Lipids constituents from *Gardenia aqualla* Stapf & Hutch

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Abstract: *Gardenia aqualla* a plant of the Rubiaceae family is being used extensively in Africa, particularly in Cameroon as an herbal medicine. Therefore it is necessary to have knowledge of the constituents of the plant of our native species. Thus, the aim of the present study was to investigate chemical constituents of this herbal medicine. Nine compounds, including one alkane, n-Nonacosane (**1**), two aliphatic alcohols n-heptatriacontanol (**2**) and n-Docosanol (**3**), one fatty ester, Heptadecyl heptacosanoate (**4**), two sugars, D-mannitol (**5**) and D-mannitol acetate (**6**), and a mixture of three phytosterols, *β*-sitosterol (**7**), stigmasterol (**8**) and fucosterol (**9**), have been isolated and purified from the stem barks of *Gardenia aqualla* Stapf & Hutch. Their structures were elucidated using spectroscopic analyses, including 1D and 2D NMR and ESI-MS. The fatty acid ester, heptadecyl heptacosanoate (**4**) is reported here for the first time. All the isolated compounds were tested for their antimicrobial activities against four Gram negative bacteria (*Salmonella Typhimurium* ATCC6539, *Pseudomonas aeruginosa* ATCC9721, *Escherichia coli*, and *S. Typhi*) and four yeasts (*Candida albicans* ATCC9002, *Candida parapsilosis* ATCC22019, *Candida krusei* and *C. albicans*).

Keywords: *Gardenia aqualla* stem barks; Alkanes; Alkohols; Fatty acid esters; Antimicrobial.

1 Introduction

In Africa, much like elsewhere in the whole world, plants have always been an important source of natural products with very high therapeutic values. Many people are still interested in using natural products from plants for curative and preventive medicine. Thus, it has been estimated that over 60% of the world population and 80% of the population of developing countries still directly rely on medicinal plants, for their primary health care needs [1]. Hostettmann et al. [2] stated that in some African countries, up to 90% of the population still depended exclusively on medicinal plants as a source of medicines. Nowadays, people are still very interested in medicinal plants. Thus, with this growing interest in the field of medicinal plants, there is a great need to produce databases that would contain as much information as possible about the secondary metabolites produced by plants and their importance in today's world. About 15% of the known angiosperm species in tropical regions have been examined for their pharmacological properties [3]. Therefore, there are most definitely a large number of plant-derived medicines and other useful compounds that have yet to be discovered and characterized around the world.

Cameroon has a very rich and diverse flora estimated in 2003 at 8,620 known plants species [4], that a large majority of population are using some of them as medicines for their primary healthcare. However, in the Cameroonian pharmacopoeia, there is still a serious lack of information on the uses and the phytochemical content of a large number of plants and spices traditionally employed in

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the treatment of several ailments. *Gardenia aqualla* Stapf & Hutch (Rubiaceae) is among those medicinal plants extensively used in Cameroon and not well documented.

Known as "*Dingale*" in *Fufulde* in the Adamawa Region of Cameroon, *Gardenia aqualla* is a bushy shrub or a small tree that grows up to 3 m high and belongs to the Sudanese to Sudano-Guinean savannahs on shady lowlands and alluvial terraces [5]. It is widely distributed from Senegal to Cameroon and as far as Sudan, scattered, locally common. Medicinally, the plant is used in the treatment of several ailments such as leprosy, oral and ear infections [6], dysmenorrhea [7], jaundice, ulcers [8], diabete [9], syphilis [10], cancer [11].

As the plant is being used extensively in our country as an herbal medicine, it is necessary to have knowledge of the constituents of the plant of our native species. Previous phytochemical investigation on this species revealed the presence of steroids, triterpenes and flavonoids in the petroleum ether extract of the stem barks while the methanolic extract was found to contain anthraquinones, carbohydrates, cardiac glycosides, flavonoids, saponins, steroids, tannins and triterpenes [12]. To the best of our knowledge, currently no compound has been isolated from this plant species. The present study aims therefore to investigate chemical constituents of this herbal medicine.

2 Experimental Procedures

2.1 Plant material

The Stem barks of *G. aqualla* Stapf & Hutch were collected at Dang in the District of Ngaoundere III, Region of Adamawa Cameroon. A voucher is deposited at National Herbarium of Cameroon in Yaounde under number 36894/HNC.

2.2 Extraction and isolation

The ground dried stem barks (1.00 kg) were consecutively extracted with Hexane, EtOAc and MeOH to give 6.74 g, 9.42 g and 83.83 g of extracts respectively. A portion (50.0 g) of the MeOH extract was fractionated by silica gel column chromatography using a gradient elution with successively Hexane-EtOAc (1:0→0:1) and EtOAc-MeOH (1:0→0:1). 510 sub-fractions of 300 mL were collected and according to their chromatographic profiles on TLC, they were grouped into six fractions G_1-G_6. From the main column seven compounds were obtained, compound **1** at Hexane-EtOAc (9:1) from the assembly of sub-fractions 17-53, compound **2** at Hexane-EtOAc (1:1) from the assembly of sub-fractions 242-253, compound **5** at Hexane-EtOAc (1:9) from the assembly of sub-fractions 321-366 and compound **6** at EtOAc-MeOH (9:1) from the assembly of sub-fractions 400-402. Fraction G_3 (1.96 g) was further purified on CC of silica gel using a gradient polarity of hexane-EtOAc (1:0→6:4) leading to compound **3** from the sub-fractions 17-18, compound **4** from the sub-fractions 21-22, and from the assembly of sub-fractions 25-28 compounds **7**, **8** and **9** were obtained as a mixture. All these last five compounds were obtained at the same polarity (Hexane-EtOAc (9:1)).

Nonacosane (**1**): white solid, m.p. 50–51°C. TOF-MS-ESI$^+$: $[M+H]^+$ at *m/z* = 409.3, $[M+K]^+$ at *m/z* = 447.3, and [2M+K+H]+ at *m/z* = 856.5 for $C_{29}H_{60}$. ^{1}H NMR ($CDCl_3$): δ 0.91 (6H, t, *J* = 7.0 Hz, 3H-1, 3H-29), 1.59 (4H, m, 2H-2, 2H-28) and 1.20-1.39 [$(CH_2)_n$, *brs*]. ^{13}C NMR ($CDCl_3$): δ 14.1 (C-1, C-29), 22.7 (C-2, C-28), 31.9 (C-3, C-27), and 29.4–29.7 (C-4–C-26) [13].

Heptatriacontanol (**2**): white solid, m.p. 88–89°C. TOF-MS-ESI$^+$: $[M+H]^+$ at *m/z* = 537.3, for $C_{37}H_{76}O$. ^{1}H NMR ($CDCl_3$): δ 3.65 (2H, m, 2H-1), 1.60 (2H, m, 2H-2), 1.20-1.39 [$(CH_2)_n$, *brs*] and 0.89 (3H, t, *J* = 7.5 Hz, 3H-37). ^{13}C NMR ($CDCl_3$): δ 63.1 (C-1), 32.8 (C-2), 25.7 (C-3), 29.4–29.7 (C-4–C-34), 31.9 (C-35), 22.7 (C-36) and 14.1 (C-37). [14]

Docosanol (**3**): white solid, m.p. 58–59°C. TOF-MS-ESI$^+$: $[M+6NH_4^+{+}6H]^+$ at *m/z* = 440.2 and $[M+4NH_4^+{+}3H]^+$ at *m/z* = 401.4, for $C_{22}H_{46}O$. ^{1}H NMR ($CDCl_3$): δ 3.67 (2H, t, *J* = 6.6 Hz, 2H-1), 1.59 (2H, m, 2H-2), 1.29 [$(CH_2)_n$, *brs*] and 0.91 (3H, t, J = 7.0 Hz, 3H-22). ^{13}C NMR ($CDCl_3$): δ 63.1 (C-1), 32.8 (C-2), 25.7 (C-3), 29.4–29.7 (C-4–C-19), 31.9 (C-20), 22.7 (C-21) and 14.1 (C-22). [15]

Heptadecyl heptacosanoate (**4**): white solid, m.p. 65–66°C. TOF-MS-ESI$^+$: $[M+2NH_4^+{+}H]^+$ *m/z* = 685.3 for $C_{44}H_{88}O_2$. ^{1}H NMR ($CDCl_3$): δ 0.81 (6H, t, *J* = 7.0 Hz, 3H-27, 3H-17′), 1.20-1.33 [$(CH_2)_n$, *brs*], 1.54 (4H, m, 2H-3, 2H-2′), 2.22 (2H, t, *J* = 7.5 Hz, 2H-2), 4.00 (2H, t, *J* = 6.7 Hz, 2H-1′). ^{13}C NMR ($CDCl_3$): δ 174.0 (C-1), 64.4 (C-1′), 34.4 (C-2), 31.9 (C-25 and C-15′), 29.2-29.7 (C-4–C-24 and C-4′–C14′), 28.7 (C-2′), 26.0 (C-3′), 25.1 (C-3), 22.7 (C-26 and C-16′) and 14.1 (C-27 and C-17′).

D-mannitol (**5**): white solid, m.p. 168–169°C. TOF-MS-ESI$^+$: $[2M+Na]^+$ at *m/z* = 387.2 for $C_6H_{14}O_6$. ^{1}H NMR (DMSO-d_6): δ 4.41 (2H, d, *J* = 5.5 Hz, HO-2 and 5), 4.33 (2H, t, *J* = 5.7 Hz, HO-1 and 6), 4.13 (2H, d, *J* = 7.1 Hz, HO-3 and 4), 3.62 (2H, ddd, *J* = 10.8, 5.7, 3.5 Hz, H-1a and 6a), 3.56 (2H, t, *J* = 7.5 Hz, H-3 and 4), 3.47 (2H, m, H-2 and 5) and 3.39 (2H, m, H-1b and 6b). ^{13}C NMR (DMSO-d_6): δ 63.8 (C-1 and 6), 71.3 (C-2 and 5) and 69.7 (C-3 and 4). [16]

D-mannitol acetate (**6**): white solid, m.p. 123–124°C. TOF-MS-ESI$^+$: $[M+Na]^+$ at *m/z* = 247.1 and $[M+Na]^+$ at *m/z* = 471.1 for $C_8H_{16}O_7$. ^{1}H NMR (DMSO-d_6): δ 4.75 (1H, d, *J* = 5.6 Hz, HO-2), 4.41 (1H, d, *J* = 5.5 Hz, HO-5), 4.33 (1H, t, *J* = 5.7 Hz, HO-6), 4.28 (2H, *ov*, H-1a and HO-3), 4.14 (2H, d, *J* = 7.1

Hz, HO-4), 3.96 (1H, m, H-1b), 3.68 (1H, m, H-2), 3.62 (1H, m, H-6a), 3.54 (2H, m, H-3 and 4), 3.47 (1H, m, H-5) and 3.40 (1H, m, H-6b), 2.03 (3H, s, OCOC$\underline{H}_3$). ^{13}C NMR (DMSO-d_6): δ 20.8 (-OCO$\underline{C}H_3$), 63.8 (C-6), 67.0 (C-1), 68.2 (C-2), 69.3 (C-3), 69.4 (C-4), 71.2 (C-5) and 170.5 (-O$\underline{C}$OCH$_3$).

β–sitosterol (**7**): white solid. ^{1}H-NMR ($CDCl_3$), δ: 5.35 (1H, d, J = 5.1 Hz, H-6), 3.53 (1H, ddd, J = 15.9, 11.0, 4.6 Hz, H-3), 1.04 (3H, s, H-19), 1.01 (3H, d, J = 7.0 Hz, H_3-27), 0.95 (3H, d, J = 6.6 Hz, H_3-26), 0.88 (3H, d, J = 1.8 Hz, H_3-21), 0.85 (3H, *ov*, H_3-29) and 0.71 (3H, s, H_3-18). ^{13}C-NMR ($CDCl_3$): δ 37.3 (CH_2, C-1), 31.9 (CH_2, C-2), 71.8 (CH, C-3), 42.4 (CH_2, C-4), 140.8 (C-5), 121.7 (CH, C-6), 31.7 (CH_2, C-7), 31.9 (CH, C-8), 50.2 (CH, C-9), 36.6 (C, C-10), 21.1 (CH_2, C-11), 39.8 (CH_2, C-12), 42.3 (CH, C-13), 56.8 (CH, C-14), 24.3 (CH_2, C-15), 28.2 (CH_2, C-16), 56.1 (CH, C-17), 11.9 (CH_3, C-18), 19.0 (CH_3, C-19), 36.2 (CH_2, C-20), 18.8 (CH_3, C-21), 34.0 (CH_2, C-22), 26.3 (CH_2, C-23), 45.9 (CH, C-24), 29.3 (CH, C-25), 19.4 (CH_3, C-26), 19.8 (CH_3, C-27), 23.1 (CH_2, C-28), 12.0 (CH_3, C-29). [17]

Stigmasterol (**8**): white solid. ^{1}H NMR ($CDCl_3$), δ: 5.38 (1H, m, H-6), 5.14 (1H, *ov*, H-22), 5.05 (1H, dd, J = 15.2, 8.7 Hz, H-23), 3.56 (1H, ddd, J = 15.8, 11.0, 4.5 Hz, H-3), 1.04 (3H, s, H-19), 1.01 (3H, d, J = 7.0 Hz, H_3-27), 0.95 (3H, d, J = 6.6 Hz, H_3-26), 0.88 (3H, d, J = 1.8 Hz, H_3-21), 0.85 (3H, *ov*, H_3-29) and 0.71 (3H, s, H_3-18). ^{13}C NMR ($CDCl_3$) δ: 37.2 (CH_2, C-1), 31.9 (CH_2, C-2), 71.8 (CH, C-3), 42.3 (CH_2, C-4), 140.8 (C, C-5), 121.7 (CH, C-6), 31.7 (CH_2, C-7), 31.9 (CH, C-8), 50.2 (CH, C-9), 36.5 (C, C-10), 21.1 (CH_2, C-11), 39.8 (CH_2, C-12), 42.3 (C, C-13), 56.8 (CH, C-14), 24.3 (CH_2,C-15), 28.2 (CH_2, C-16), 56.1 (CH, C-17), 12.1 (CH_3, C-18), 19.0 (CH_3, C-19), 40.5 (CH_2, C-20), 18.8 (CH_3, C-21), 138.4 (CH, C-22), 129.3 (CH, C-23), 51.2 (CH, C-24), 45.9 (CH, C-25), 19.4 (CH_3, C-26), 19.8 (CH_3,C-27), 24.3 (CH_2, C-28), 12.3 (CH_3, C-29). [18]

Fucosterol (**9**): white solid. ^{1}H NMR ($CDCl_3$), δ: 5.38 (1H, m, H-6), 5.17 (1H, *ov*, H-28), 3.56 (1H, ddd, J = 15.8, 11.0, 4.5 Hz, H-3), 1.57 (3H, *ov*, H-29), 1.04 (3H, s, H-19), 1.01 (3H, d, J = 7.0 Hz, H_3-27), 0.95 (3H, d, J = 6.6 Hz, H_3-26), 0.88 (3H, d, J = 1.8 Hz, H_3-21) and 0.71 (3H, s, H_3-18). ^{13}C NMR ($CDCl_3$) δ: 37.2 (CH_2, C-1), 31.9 (CH_2, C-2), 71.8 (CH, C-3), 42.3 (CH_2, C-4), 140.8 (C, C-5), 121.7 (CH, C-6), 31.7 (CH_2, C-7), 31.9 (CH, C-8), 50.2 (CH, C-9), 36.5 (C, C-10), 21.1 (CH_2, C-11), 39.8 (CH_2, C-12), 42.3 (C, C-13), 56.8 (CH, C-14), 24.3 (CH_2,C-15), 28.2 (CH_2, C-16), 56.1 (CH, C-17), 11.8 (CH_3, C-18), 19.3 (CH_3, C-19), 36.5 (CH_2, C-20), 18.8 (CH_3, C-21), 36.1 (CH, C-22), 26.1 (CH, C-23), 145.9 (CH, C-24), 34.0 (CH, C-25), 21.1 (CH_3, C-26), 21.1 (CH_3,C-27), 116.4 (CH_2, C-28), 12.8 (CH_3, C-29). [19]

2.3 Antimicrobial assays

Antibacterial and anticandidal activities of isolated compounds were performed against four bacterial strains (*Salmonella Typhimurium* ATCC6539, *Pseudomonas aeruginosa* ATCC9721, *Escherichia coli*, and *S. Typhi* isolate) and four strains of yeast (*Candida albicans* ATCC9002, *Candida parapsilosis* ATCC22019, *Candida krusei* and *Candida albicans* isolate). Minimum inhibitory concentrations (MICs), minimum bactericidal concentrations (MBCs) and minimum fungicidal concentrations (MFCs) were determined by the broth microdilution method as previously described by Nyemb et al. [20] and Dzoyem et al. [21] respectively for antibacterial and antifungal activities. Ciprofloxacin was used as standard drug for antibacterial assay, while ketoconazole was used as positive control for the antifungal assay. All the experiments were carried out in triplicate.

Ethical approval: The conducted research is not related to either human or animals use.

3 Results and Discussion

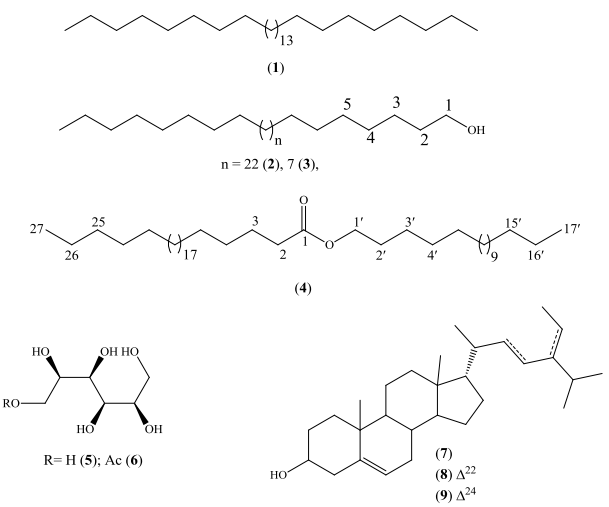

Figure 1: Isolated compounds from *G. aqualla* stem barks.

Compound **4** was obtained as a white powder. Its molecular formula was found to be $C_{44}H_{88}O_2$ on the basis of its TOF-MS-ESI$^+$ spectra that showed pseudo-molecular ion peak $[M+2NH_4^+ + H]^+$ at m/z = 685.3. The ^{1}H NMR had peaks characteristics of aliphatic esters [22-24]. The spectrum revealed the presence of a signal of two protons triplet at δ_H 4.00 (2H, t, J = 6.7 Hz) probably due to the 2H-1′ deshielded by the proximity of the ester function fixed on the same carbon C-1′, a broad signal between δ_H 1.20 and 1.33 was attributed to the hydrocarbon chain (CH_2)n, this signal showed an integration of 74 protons corresponding to 37 methylene groups. A triplet of six protons at δ_H 0.81 (6H, t, J = 7.0 Hz) corresponding to two terminal

methyl groups was also visible. The ^{1}H NMR spectra also displayed a signal of two protons triplet at δ_H 2.22 (2H, t, J = 7.5 Hz) attributed to the methylene protons adjacent to the carbonyl group of the ester function. The ^{13}C NMR spectral data indicated characteristic signals of a fatty ester among which a signal of a carbonyl ester function at δ_C 174.0 attributable to the C-1, a signal of a methylene carbon at δ_C 64.3 (C-1′) deshielded by the proximity of the ester function, a signal at δ_C 14.1 corresponding to the terminal methyl groups (C-27 and C-17′). The remaining methylene carbons appeared between δ_C 22.7 and 31.9 were assigned accordingly. The COSY spectra of compound **4** revealed one spin system associated with the alkane long chain. Thus, the methyls at δ_H 0.81 (6H, t, J = 7.0 Hz) correlated with a set of protons at δ_H 1.20 which in turn correlated with the methylene protons at δ_H 1.54 (*m*). The correlations of the methylene at δ_H 1.54 with the ones at δ_H 2.22 (2H, t, J = 7.5 Hz) and 4.00 (2H, t, J = 6.7 Hz) respectively were also visible (Figure 2). From the HMBC spectra of compound **4**, long range correlations were unambiguously detected between the protons 2H-1′ at δ_H 4.00 and carbons C-2′ (δ_C 28.4), C-3′ (δ_C 26.0) and C-1 (δ_C 174.0) ; methylene protons 2H-2′ (δ_H 1.54) and carbons C-1′ (δ_C 64.3), C-3′ (δ_C 26.0), C-4′ (δ_C 29.1) and C-1 (δ_C 174.0). Another set of long range correlations was observed between the methylene at δ_H 2.22 (2H-2) and C-1 (δ_C 174.0), C-3 (δ_C 25.1) and C-4 (δ_C 29.2) (Figure 2). On the basis of above discussion the structure of compound **4** was elucidated as heptadecyl heptacosanoate. The mass spectral fragmentation was also consistent with the proposed structure. The prominent ion fragments arising at *m/z* 393.3 $[M\text{-}C_{17}H_{35}O^{\bullet}]^{+}$ and 409.2 $[M\text{-}C_{17}H_{35}O_2^{\bullet}]^{+}$ (Figure 3) suggested that heptacosanoic acid was esterified with heptadecanol. The fragment ion arising at *m/z* 315.2 $[C_{19}H_{38}O_2{+}NH_4{+}\text{-}H]^{+}$ correspond to the loss of hexacosene ($C_{26}H_{52}$) through a Mc Lafferty rearrangement. Further loss of 113 uma ($C_8H_{17}^{\bullet}$) by this fragment led to the base peak at *m/z* 185.0.

Nonacosane (**1**), heptatriacontanol (**2**), Docosanol (**3**), D-mannitol (**5**), D-mannitol acetate (**6**), and a mixture of *β*-sitosterol (**7**), stigmasterol (**8**) and fucosterol (**9**) in a *ratio* of 3:1:2 (estimated from the relative intensities of protons H-22 and H-23 of stigmasterol (8), H-28 of fucosterol (9) and H-6 in ^{1}H-NMR signals), were also isolated and characterized from spectral analysis and comparison with the literature [13-19].

The antibacterial and anticandidal activities of compounds which were in sufficient amount were investigated using the broth microdilution method. This microdilution method has been adopted because it is less expensive, less cumbersome than the macrodilution method and it yields reproducible results. Bacterial

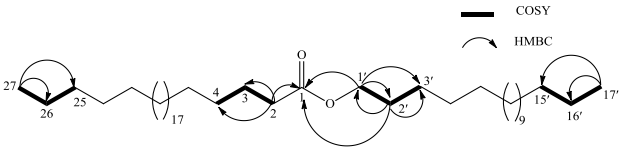

Figure 2: Important COSY and HMBC correlations of compound **4.**

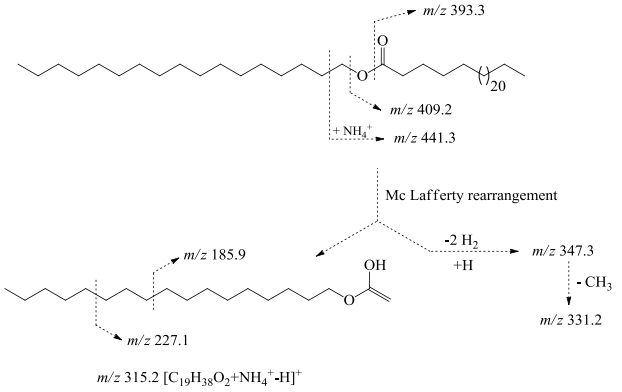

Figure 3: Important fragmentation of compound **4.**

growth could be assessed either visually by grading turbidity or spectrophotometrically by measuring optical density. The MICs and MFCs of all the tested compounds were determined (Table 1). The tested compounds showed variable antimicrobial activity with MICs values ranging from 32 to 128 µg/mL. D-mannitol (**5**) and D-mannitol acetate (**6**) presented the highest antibacterial activities depending on the bacteria strains, while they were inactive (> 128 µg/mL) against all tested yeast strains. These two compounds had the same activities (MIC = 32 µg/mL) against the isolate strains of *E. coli* and *S. typhi*. This activity has decreased for D-mannitol acetate (**6**) against *S. typhi* ATCC6539 (MIC = 128 µg/mL), and increased against *P. aeruginosa* ATCC972. The highest antifungal activity (MIC = 128 µg/mL) was recorded for docosanol (**3**) and the mixture of phytosterols *β*-sitosterol (**7**), stigmasterol (**8**) and fucosterol (**9**) against all the tested yeast except for *C. parapsilosis* which was not sensitive to the mixture of phytosterols.

4 Conclusion

This study conducted on the stem barks of *Gardenia aqualla*, is a part of our research on the bioactive constituents of the medicinal plant of Cameroon pharmacopeae. *G. aqualla* is a medicinal plant growing in the Sudano-Guinean savannahs of the country. From the MeOH extract of the plant stem barks, nine compounds were isolated and their structures were elucidated by

Table 1: MICs, MBCs and MFCs of some isolated compounds.

		Tested compounds and MIC, MBC and MFC values (µg/mL)											
Microorganism		**3**		**4**		**5**		**6**		**7+8+9**		**Ciprofloxacin**	
Bacteria		**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
E. coli	Isolate	128	-	128	-	64	128	64	-	128	-	0.5	0.5
	ATCC6539	-	-	128	-	32	128	128	-	128	128	0.5	1
S. typhi	Isolate	128	-	128	-	32	32	32	-	-	-	0.5	0.5
P. aeruginosa	ATCC9721	128	128	128	128	128	128	64	128	-	-	0.5	1
Yeasts		**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**	**ketoconazole**	
C. parapsilosis	ATCC22019	128	-	-	-	-	-	-	-	-	-	2	16
	ATCC9002	128	-	-	-	-	-	-	-	128	-	0.5	64
C. albicans	Isolate	128	-	-	-	-	-	-	-	128	-	0.25	64
C. krusei	Isolate	128	-	-	-	-	-	-	-	128	-	2	64

(-): >128 µg/mL. *E. coli*: *Escherichia coli*; *S. typhi*: *Salmonella Typhimurium*; *P. aeruginosa*: *Pseudomonas aeruginosa*; *C. parapsilosis: Candida parapsilosis; C. albicans: Candida albican; C. krusei: Candida krusei*

extensive NMR spectroscopy and Mass Spectrometry. This is the first report on the isolation of compounds from this plant species and to the best of our knowledge, compound **4** is reported here for the first time from plant kingdom, while compounds **1**, **2**, **3**, **6** and **9**, are reported here for the first time from the genus *Gardenia*.

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Conflict of interest: Authors state no conflict of interest.

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